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The mitochondrial genomes of *Campodea fragilis* and *C. lubbocki* (Hexapoda: Diplura): high genetic divergence in a morphologically uniform taxon

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Abstract

Mitochondrial genomes from two dipluran hexapods of the genus *Campodea* have been sequenced. Gene order is the same as in most other hexapods and crustaceans. Secondary structures of tRNAs reveal specific structural changes in tRNA-C, tRNA-R, tRNA-S1 and tRNA-S2. Comparative analyses of nucleotide and amino acid composition, as well as structural features of both ribosomal RNA subunits, reveal substantial differences among the analysed taxa. Although the two *Campodea* species are morphologically highly uniform, genetic divergence is larger than expected, suggesting a long evolutionary history under stable ecological conditions.

Introduction

Mitochondrial sequence data are commonly used in phylogenetic studies from large scale phylogenetics down to the population level. With few exceptions, mitochondrial genomes from multicellular animals are circular DNA molecules, about 16 Kb in size, containing 37 genes encoding for two ribosomal RNA molecules (*rrnS* and *lrrS*), 13 protein subunits (*cox1-3*, *cob*, *nad1-6* and *nd4L*, *atp6* and *atp8*) and 22 transfer RNA molecules (*trnX*) *(*Wolstenholme, 1992; Boore, 1999). In addition, mitochondrial genomes of insects bear one large A+T rich noncoding region, which is involved in the initiation of mitochondrial replication and transcription (Wolstenholme, 1992), and is therefore commonly referred to as the mitochondrial control region.

Because of the ease of amplification and sequencing, the mitochondrial genome has been extensively studied for phylogenetic purposes, as well as for its peculiar patterns of molecular evolution (Boore, 1999). Concerning the latter, the mitochondrial genome has been found to exhibit extensive nucleotide compositional bias. One of the best examples of mitochondrial compositional bias is that observed in insects (Simon *et al.*, 1994), and some other arthropods (e.g.: Black *et al.*, 1998; Negrisolo *et al.*, 2004), where a significant higher amount of Adenine and Thymine nucleotides (A+T bias) has been found. Another type of compositional bias is that affecting base content at Protein Coding Genes (PCGs) in the different strands of the mitochondrial genome (Perna & Kocher, 1995). In a thorough investigation of this strand-specific bias across Metazoa, Hassanin *et al.* (2005) found clear evidence of an asymmetric mutational bias, as well as traces of the reversal of such a bias in some taxa due to an inversion of the control region.

Gene rearrangements of the mitochondrial genome are also well known, and several mechanisms have been proposed to explain gene order changes, involving duplication events followed by the random (Boore, 2000) or non-random (Lavrov *et*

al., 2004) loss of some duplicated copies. These events sometimes leave unusually large fragments of non-coding DNA, in addition to the canonical control region (Lavrov *et al.*, 2002).

While partial sequences from the control region or from single genes, like *cox1*, *cob*, *rrnS*, have been utilised in phylogenetic studies at the species or population level, complete mitochondrial genome data have been used predominantly in phylogenetic analyses above the family level (e.g: Nardi *et al.*, 2003; Negrisolo *et al.*, 2004; Cameron *et al.*, 2005; Cook *et al.*, 2005). In most of the latter cases only PCGs were studied. However, complete mitochondrial genomes provide more data than just sequence information:

(1) Gene order is not conserved among metazoa and gene translocations may serve as characters in phylogenetic analyses. For example the “Pancrustacea” hypothesis - a close relationship between Crustacea and Hexapoda (excluding the Myriapoda, which were traditionally viewed as sistergroup to hexapods) - has been suggested on the basis of a gene translocation involving a mitochondrial tRNA gene (Boore *et al*., 1998).

(2) Secondary structure features of tRNAs and rRNAs are seldomly used as characters in phylogenetic analyses but with a growing amount of data these may be of greater value soon (e.g. Macey *et al.*, 2000).

(3) The mitochondrial genetic code differs in some codons from the standard code and also varies among metazoan taxa. These deviations are of phylogenetic value as well (e.g. Castresana *et al*., 1998; Telford *et al.*, 2000).

During the last ten years the traditional view of arthropod phylogeny has been strongly challenged by molecular analyses. As a result the Pancrustacea hypothesis is now well supported by mitochondrial gene rearrangement data (Boore *et al.*, 1998) and sequence based analyses of both, mitochondrial (Wilson *et al.* 2000; Hwang et al. 2001; Lavrov *et al.*, 2004) and nuclear genes (Mallatt *et al.*, 2004, Regier *et al.*,

2005). On the other hand, crustacean and even hexapod monophyly, as well as their internal relationships, are still under debate (Regier *et al.*, 2005; Babbitt & Patel, 2005; Podsiadlowski & Bartolomaeus, 2005). In particular, some analyses using complete mitochondrial datasets do not support the monophyly of Hexapoda, with different crustacean subtaxa forming sistergroups to Collembola and Insecta (Nardi et al., 2003; Cook *et al.*, 2005). In this context, apterygote taxa play a crucial role, and their reciprocal relationships have not yet been resolved with certainty (Kristensen, 1997; Kukalova-Peck, 1987; Stys & Bilinski, 1990; Carapelli et al., 2000). Recently, the first mitochondrial genome of a member of Diplura, the japygid *Japyx solifugus*, has been published (Carapelli *et al.*, 2005) and the corresponding phylogenetic analysis suggests a paraphyletic origin of Hexapoda. On the other hand detailed analyses of hexapod relationships based on the nuclear 18S rRNA results in hexapod monophyly (Kjer, 2004; Babitt & Patel, 2005). The increased use of rRNA sequences in phylogenetic studies has stimulated the debate over the correct alignment procedures. One approach calls for using the information contained in rRNA secondary structures (Kjer, 1995, 2004), which, in turn, are inferred from general models based on the comparison of a large number of sequences (Hickson *et al.*, 1996).

In this study we present two additional mitochondrial genomes from apterygote hexapods: *Campodea fragilis* and *C. lubbocki* (Diplura: Campodeidae). We provide detailed analyses of nucleotide and amino acid frequencies, and compositional bias, as well as the reconstruction of the putative secondary structures of tRNA and rRNA genes. Although a lot of mitochondrial genomes from arthropods are published now, only a few congeneric species are compared. The genus *Campodea* has a worldwide distribution and comprises about 150 soil-dwelling, herbivorous species, sized up to 5 mm and highly uniform in appearance. The two species studied exhibit only a few morphological differences: spatulate (*C.l.*) or acuminate (*C.f.*) praetarsal

appendages; presence (*C.l.*) or absence (*C.f.*) of mediane macrochaetae on abdominal tergites; annulated (*C.l.*) or smooth (*C.f.*) cerci (Palissa, 1964). Both seem to occupy similar ecological niches but their genetic divergence turned out to be rather high, suggesting a long evolutionary history under stable environmental conditions.

Results and Discussion

Genome organisation

Both *Campodea* mitochondrial genomes presented here share the same 37 genes with most bilaterian animals and have the same gene arrangement (Fig. 1) as other apterygote hexapods (*Tricholepidion gertschi*, NC005437; *Nesomachilis australica*, NC006895; *Japyx solifugus* AY771989), insects (*Drosophila melanogaster* NC001709), and crustaceans (*Daphnia pulex* NC000844; *Penaeus monodon* NC002184). This gene order appears to be the ancestral state for Pancrustacea (Crustacea+Hexapoda) and differs from that of chelicerates and myriapods by the translocation of *trnL1* (Boore *et al*., 1998).

In both *Campodea* species gene overlaps exist between the PCGs *atp8/atp6* (7 nucleotides) and *nad4/nad4L* (7 nucleotides). Other overlaps occur between tRNA genes or between a tRNA- and a protein-coding gene. Only a few overlaps are larger than 3 nucleotides, notably at the *trnY*/*cox1* boundary in both species (8nt in *C. lubbocki* and 5nt in *C. fragilis*). A large non-coding region is present between *rrnS* and *trnI* (558nt in *C. fragilis* and **561**nt in *C. lubbocki*). This is, by all evidence available, homologous to the mitochondrial control region (CR), which, in insects, is usually called AT-rich region for exhibiting a remarkable A+T-bias (up to >90% in hymenopterans; Crozier & Crozier, 1993). In *Campodea,* the putative AT-rich region

shows A+T content between 84.2% (*C. fragilis*) and **89.1%** (*C. lubbocki*). More unusual, other non-coding (*ncod*) sequences are found between other mitochondrial genes of the two *Campodea* species. One of them, shared by both species, is located between *trnS2* and *nad1* (56nt in *C. fragilis*, 80nt in *C. lubbocki*), while only *C. fragilis* has an additional *ncod* region of 111nt between *nad2* and *trnW* (Fig. 1). Interestingly, the two *ncod* regions can be folded in apparently stable secondary structures (Fig. 2). Most other arthropods lack non-coding sequences larger than a few nucleotides (with the control region as an exception). Although very unusual, the occurrence of large non-coding regions abutting gene junctions has been observed in insects and myriapods (Lavrov *et al*., 2002; Bae *et al*., 2004). Sometimes these fragments are mentioned as remnants of gene duplication events due to possible errors during the replication of the mitochondrial genome (Boore, 2000). Extra copies of mitochondrial genes are subsequently eliminated from the compactly arranged mitochondrial genome (Boore, 1999). The occurrence of these structures would suggest that the remnant of an exceeding copy of a tRNA gene is still present in the genomes of the taxa under study. In addition, the *ncod* fragments found in both *Campodea* species between *trnS2* and *nad1* clearly exhibit homologous patterns in either primary sequence and secondary structure. Accordingly, this feature would suggest that the presumed duplication event has occurred before the speciation of the two taxa.

A+T content of complete mitochondrial genomes varies among insects (Table 1). While *Japyx solifugus* (Japygidae), the only other dipluran species with published mitogenomic data, has the lowest value among hexapods (64.8%), *C. lubbocki* has the highest value among apterygote insects (74.9%), followed by two collembolans (*Gomphiocephalus hodgsoni* and *Tetrodontophora bielanensis*) and *C. fragilis* (72.6%). Comparatively, high values are also found in Pterygota, while archaeognathan and zygentoman species range between 67-69%. In all species,

third-codon position sites of PCGs have significantly higher A+T content than either first and second codon positions. Functional reasons for the strong differences of A+T content among hexapods are still under debate.

Protein coding genes

There is no significant difference in the size of the PCGs of *C. fragilis* and *C. lubbocki* compared to each other and to other apterygote hexapods (Table 2). The three starting codons ATA, ATG and ATT are used in both species, while only in *C. lubbocki* the starting codon ATC is used for *atp8*, *nad1* and *nad5*. In some cases stop codons are truncated (*cox1-3* in *C. fragilis*; *cox3*, *nad3, nad4* and *nad6* in *C. lubbocki*) and possibly post-transcriptionally completed to TAA after cleavage (Okimoto *et al*., 1990, Lavrov *et al*., 2002).

Nucleotide sequence similarity of PCGs between the two *Campodea* species ranges from 49.5% (*nad6*) to 77.9% (*cox2*) (Table 2). As an example, the pairwise similarity of *cox1-cox3* between *C. fragilis* and *C. lubbocki* (77.1%) is rather low: in the same region, four *Ixodes* species show levels of nucleotide divergence ranging from 75% to 82%, five *Drosophila*-species are in the range 92-99%, while two species of the crustacean *Triops* (T. *cancriformis* and *T. longicaudatus*) differ by 81.2%, and two species of *Bombyx* (*B. mori* and *B. mandarina*) by 96%. In contrast, the divergence between the two *Campodea* sequences is more similar to that observed between species from different orders, such as in the comparison between *Pteronarcys princeps* (Pterygota: Plecoptera) and five *Drosophila* species (78-79%), or the comparison of *P. princeps* with the zygentoman *Tricholepidion gertschi* (73.5%). Frati *et al.* (1997) provided a comparison between different collembolan species and other hexapods based on *cox2* sequences. Levels of nucleotide similarity for *cox2* between congeneric species (genera *Orchesella* and *Isotomurus*) were only slightly higher (80.4-81%) than that observed between *C. fragilis* and *C. lubbocki* (**77.9% or**

78.5%?). On the other hand, nucleotide similarity of *cox2* between *Campodea* species and *J. solifugus* varies between **67.4% and 67.8%**, well in the range of that estimated, in Collembola, between species from different lineages (61.9%-69.7%), or between collembolan species and other pterygote insects (60.4%-68.9%). Looking at the inferred amino acid sequence similarity, the values estimated in diplurans (**68.1%-69.5%** between *Campodea* and *J. solifugus*) are comparable to those found between species from different orders among pterygote insects (Simon *et al.,* 1994). We conclude that, although morphologically very uniform, the taxon *Campodea* is genetically highly diverse and that, under the molecular clock hypothesis, the split between the two species occurred comparably long ago, perhaps as long as some of the basal splits among pterygote insects. Indeed, taxonomic categories above the species do not necessarily imply similar genetic and morphological differentiation, nor similar age in different lineages. Our results in *Campodea* parellel those obtained in collembolans (Frati *et al.*, 1997, 2000) in indicating high levels of genetic divergence (in itochondrial sequences) even between congeneric species which do not exhibit comparable morphological differentiation. These results point towards either accelerated rates of evolution or remarkably older age of genera of apterygote hexapods.

As shown by Hassanin *et al.* (2005), PCGs differ for their A *vs.* T and C *vs.* G content according to the strand (J- or N-strands; *sensu* Simon *et al.*, 1994) where they are encoded. Genes encoded on the J-strand show a slight bias towards Ts over As and a strong bias towards Cs over Gs, while genes encoded on the N-strand show a much stronger bias towards Ts over As and towards Gs over Cs (Table 2, Fig. 3). One reason for this outcome might be the asymmetrical replication process, during which one strand is single-stranded for a longer time than the other, and therefore more susceptible for specific mutations (for more details see: Hassanin *et al*., 2005).

Relative synonymous codon usage in PCGs (Table 3) reveals that the genome-wide base compositional bias for A+T is also reflected in codon usage. The two *Campodea* species differ in preferred codons for Glycine, Proline and Threonine, whereas both species differ from *J. solifugus* in codon preferences for Alanine, Histidine and Valine. In the case of Histidine, this reflects the strong differences in A+T content, as in *J.solifugus* codon the codon CAC is preferred, whereas *Campodea* species prefer CAT. A similar observation is evident in the case of Leucine, for which TTA is the preferred codon in all three diplurans, but to a much stronger degree in *Campodea* than in *J. solifugus*.

Transfer-RNA coding genes

In both species all 22 tRNA-encoding genes usually found in mitochondrial genomes of metazoans are present. In both species the most likely secondary structure models for tRNA-R, tRNA-S1 and tRNA-S2 lack the D-arm (Fig. 4). In *C. lubbocki* also the tRNA-C has a shortened (or missing) D-arm. In contrast, all tRNAs mentioned above show the usual cloverleaf secondary structure in *J. solifugus* (Fig. 4), as well as all remaining tRNAs in *C. fragilis* and *C. lubbocki* (data not shown). Accordingly, a reduction of the D-arm of tRNA-R, and the two types of tRNA-S, must have evolved after the split between Campodeidae and Japygidae. Reduction of the D-arm of tRNA-C probably occured even later, after the split between *C. lubbocki* and *C. fragilis*. These data may be of value for phylogenetic inferences, when mitogenomic data will be available from more dipluran species.

Ribosomal-RNA coding genes

The two genes for ribosomal RNA subunits (*rrnL* for 16S rRNA and *rrnS* for 12S rRNA) can be folded in an inferred secondary structure composed of paired and single strand fragments (Figs. 5, 6). Comparative analysis shows that both rRNA

subunits are composed of a mosaic of variable and conserved sequence fragments, which are distributed in paired and unpaired regions of all domains (Fig. 7). This is probably due to variable functional constraints, acting at different degrees in specific parts of the rRNA subunits, which are necessary to maintain the ribosome functionality. This evidence suggests that different rates of nucleotide substitution affect each domain of *rrnL* and *rrnS*. Sequence similarity in aligned (based on secondary structure) rRNAs of *C. lubbocki* and *C. fragilis* is within the range of sequence similarity of the PCGs (*rrnL*: 74.3%; *rrnS*: 73.8%).

In general, core structural elements of *C. fragilis* and *C. lubbocki* rRNA subunits closely resemble those proposed for *Drosophila melanogaster*. The inferred secondary structure of the 12S rRNA of each species (Fig. 5) displays a considerable degree of differentiation in terms of length and shape of most of domains I and II (Fig. 7a), with conserved stretches of sequences present only on helices *h* and *m*. Conversely, domain III is probably the most conserved fragment of the entire 12S rRNA subunit, showing many invariant structural elements between *C. fragilis* and *C. lubbocki* (Figs. 5, 7a: all helices from *o* to *a1* with the exception of *u*) and some identical stretches of primary sequence. With few exceptions aside (Page, *et al.* 2002), this evidence is in agreement with the data obtained from broader comparisons that include extended datasets of animal mitochondrial 12S rRNA sequences (Hickson *et al.*, 1996; Simon *et al.*, 1996).

Domain I is probably the most variable part of the 16S rRNA subunit (Fig. 6), differing in terms of length and primary sequence between *C. fragilis* and *C. lubbocki*. On domain II the highest levels of sequence variability can be observed in the most peripheral helices (*g*, *j*, *k* and *o*), in the descendant part of helix *l* and in the single strand segments connecting helices *l, m* and *n* (Fig. 7b). An extensive primary sequence homology can be observed in the fragment enclosed between the ascendant and the descendant parts of helix *t*.

Experimental procedures

C. lubbocki; DNA isolation and PCR

Campodea lubbocki was collected in the garden of the Institute for Zoology, Freie Universität Berlin. One individual was cut into pieces and directly used for rolling circle amplification with the Templify kit (Amersham) following the manufacturer's protocol. Templify treated material was diluted with water (1:5) and used as DNA template in PCR. Two additional specimens were ethanol fixed for SEM, in order to determine the species according to Palissa (1964).

Initially five partial mitochondrial sequences (*cox1, nad5, cob, rrnL, rrnS*) were determined with PCR primer pairs designed for this purpose by looking for conserved regions of mitochondrial genes from other hexapod and crustacean sequences (Podsiadlowski & Bartolomaeus, 2005). PCR primers were purchased from metabion (Germany). PCR was performed on Mastercycler and Mastercycler Gradient (Eppendorf, Hamburg, Germany) using the Eppendorf HotMasterTaq kit. 50 µl reaction volumes were set up as follows: 42 µl sterilized distilled water, 5 µl 10x reaction buffer 1µl dNTP mix (Eppendorf) 1 µl primer mix (10 µM each), 1 µl DNA template, 0,2 µl (1u) HotMasterTaq polymerase. The cycling protocol included an initial denaturation step (94°C, 2 min), 40 cycles of denaturation (94°C, 30 sec), annealing (1 min, primer specific annealing temperature) and extension (68°C, 90 sec) and a final extension step (68°C, 1 min). After agarose (0,9%) gel separation and visualization of ethidium-bromide stained PCR products, purification for sequencing (see below) was performed using the PCR purification kit (Qiagen) or when necessary using the Gel extraction kit (Qiagen).

In a second step the determined sequences were used to design five additional PCR primer pairs bridging the gaps between them. PCR was performed as described above, except for using an extension time of seven minutes. PCR products were inspected and purified as described above.

C. fragilis; DNA isolation and PCR

Total DNA was extracted from a specimen of *C. fragilis* collected nearby the Belcaro Castle (Siena; Italia) using the Wizard SV Genomic DNA purification system (Promega). Preliminary amplification of short fragments of *nad4* and *rrnL*, obtained using mitochondrial universal primers (Simon *et al.*, 1994), were used to design species-specific primers to generate two long PCR products corresponding to the entire mitochondrial genome. The first long PCR product (about 6.5 Kb) amplified the fragment between *nd4* and *rrnL*, with the primer pair Camp-rRNAN (5'-GGTTGTATCGGAAGCTGCAGCTAG-3') and Camp-nd4J (5'-CTGGGTCGATAGTTTTGGCTGC-3'), using the following two-steps PCR conditions: 35 cycles at 96°C for 1 min and 68°C for 8 min, followed by incubation at 68°C for 15 min. The second fragment (8.5 Kb) corresponding to the sequence encompassed between *rrnL* and *nad4*, was amplified with the primer pair Camp-rRNAJ and Camp-nd4R (corresponding to the reverse complementary of the previous pair), using the following PCR condition: 35 cycles at 96°C for 1 min and 68°C for 12 min, followed by incubation at 68°C for 15 min.

PCR reactions were performed on a GeneAmp® PCR System 2700 (Applied Biosystem) in 25 µl reaction volume set up as follows: 10,75 µl of sterilized distilled water, 2.5 µl 10x reaction buffer, 2.5 µl of 25 mM $MgCl_2$, 4µl dNTP mix, 1.25 µl of each primer (10 µM), 2.5 µl DNA template and 0,25 µl (1.25u) of TaKaRa LA Taq polymerase (Takara). Each PCR reaction yielded a single band when visualized with ethidium bromide staining after electrophoresis in a 1% agarose gel.

Sequencing and sequence analysis

Sequencing of *C. lubbocki* mitochondrial DNA was carried out with a CEQ 8000XL automated DNA Analysis System (Beckman Coulter) using the CEQ DTCS Kit following the manufacturer's protocol, and using initially PCR primers and subsequently new internal primers until completion of sequences (primer walking). In *C. fragilis*, the two long PCR fragments were purified using a Montage PCR Centrifugal Filter Device (Millipore), and processed for DNA sequencing by the production facility of the DOE Joint Genome Institute (Walnut Creek, California). Flanking regions of the two long PCR sequences were then re-amplified using species-specific primers and sequenced in a CEQ 8000XL. All sequences were assembled using Sequencher 4.2.2 (Gene Codes) and the chromatograms were examined by eye to verify sequencing accuracy. The amount of clones sequenced provided a 10X average coverage for both long PCR fragments.

To determine gene identity BLAST search on NCBI Blast Entrez databases was used. Not determinable by primary sequence information alone, the presumed location of rRNA genes in both *Campodea* species has been determined according to the observed similarity in primary sequence with other hexapod taxa, and to comparison of 12S and 16S rRNA secondary structures between the two dipluran species and *D. melanogaster*. Start codons in protein coding genes were inferred to be the nearest start codon to the beginning of the sequence alignment of homologous genes with other hexapod species. Most tRNAs were identified using tRNAscan-SE 1.21 (Lowe & Eddy, 1997) and DOGMA (Wyman *et al.*, 2004), the remaining ones were found by eye inspection of the suspected regions. Transfer-RNA identity was specified by its anticodon sequence.

The secondary structures of the mitochondrial rRNA subunits (12S and 16S) for both *Campodea* species were derived by analogy with available models obtained for other

arthropods (Gutell *et al.*, 1994; Misof & Fleck, 2003; Cannone *et al.*, 2002). Secondary structures of 12S and 16S rRNA genes and of intergenic non-coding regions were then visualized using the program RnaViz 2.0 (De Rijk & DeWachter, 1997).

Nucleotide frequencies and codon usage was determined using DAMBE (version 4.2.13; Xia & Xie, 2001). PCGs were aligned and compared using Bioedit (version 7.0.1; Hall, 1999).

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Legends to Figures

Figure 1. Genome organization of the mitochondrial genomes of two *Campodea fragilis* and *C. lubbocki*. Arrows indicate direction of coding regions, numbers inside the circle represent the size of the fragments separating two adjacent genes (positive values) or the amount of shared nucleotides between two overlapping genes (negative values). Large non-coding regions are dashed, with the one located between *rrnS* and *trnI* being the putative control region.

Figure 2. Hypothetical secondary structure features found in intergenic non-coding regions: *ncod* between *trnS2* and *nad1* in *C. fragilis* (a) and *C. lubbocki* (b); *ncod* between *nad2* and *trnW* in *C. fragilis* (c).

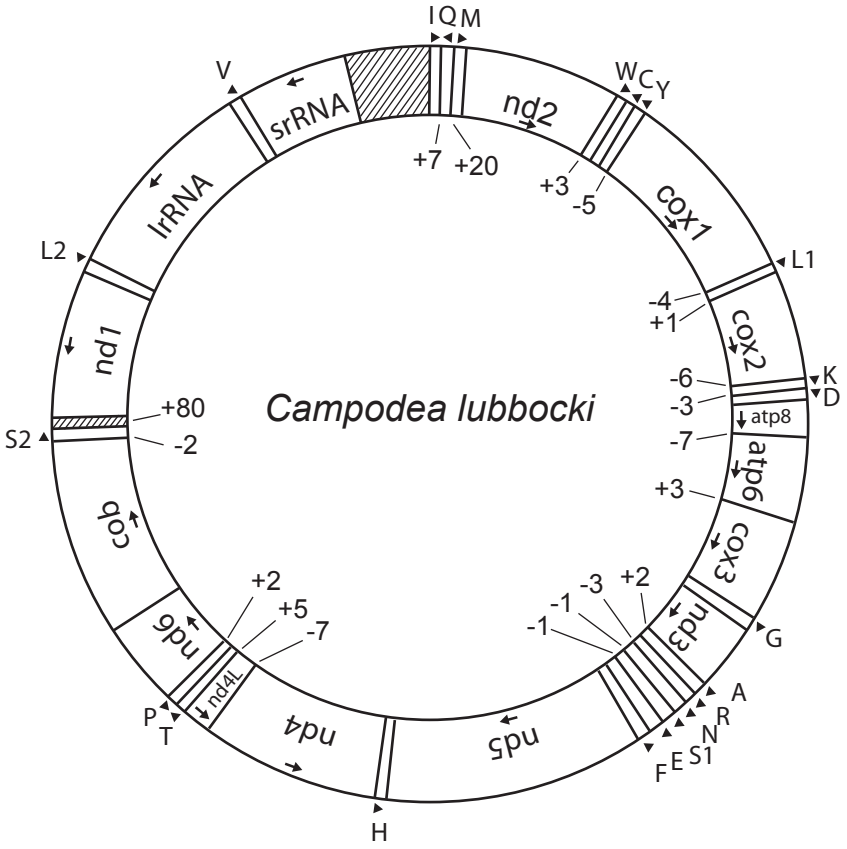
Figure 3. A+T content, calculated in a sliding window of 100 bp, along the J-strand of the mitochondrial genomes of *C. fragilis* and *C. lubbocki*. The position and coding directions of protein- and rRNA-coding genes are shown below.

Figure 4. Putative secondary structures of mitochondrial tRNAs of *C. fragilis*, *C. lubbocki*, and *Japyx solifugus*. Only tRNAs exhibiting secondary structure changes in one or both *Campodea* species are shown. All other tRNAs of *C. fragilis* and *C. lubbocki* can be folded into cloverleaf-like secondary structures.

Figure 5. Secondary structure model of the mitochondrial 12S rRNA in the two dipluran species.

Figure 6. Secondary structure model of the mitochondrial 16S rRNA in the two dipluran species.

Figure 7. Generalized secondary structure model of *Campodea* mitochondrial 12S (a, domains I-III) and 16S (b, domains I, II, IV and V) rRNAs. Grey beads show the most variable regions, in base composition, sequence length or secondary structure, of the different domains in both species. Black beads represent highly conserved fragments.


Campodea lubbocki
I Q M
W C Y
L1
K
D
G
A
R
N
S1
E
F
H
P
T
L2
S2
V
nd2
cox1
cox2
atp8
atp6
cox3
nd3
nd5
nd4
nd4L
nd6
cob
nd1
lrRNA
srRNA
+7
+20
+3
-5
-4
+1
-6
-3
-7
+3
-1
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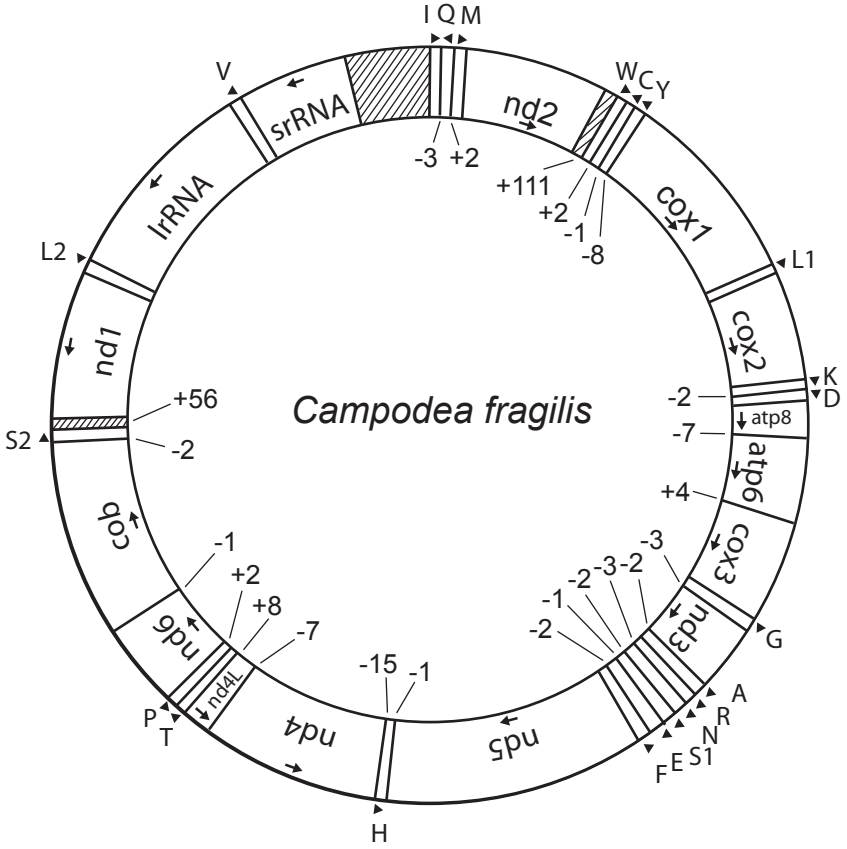
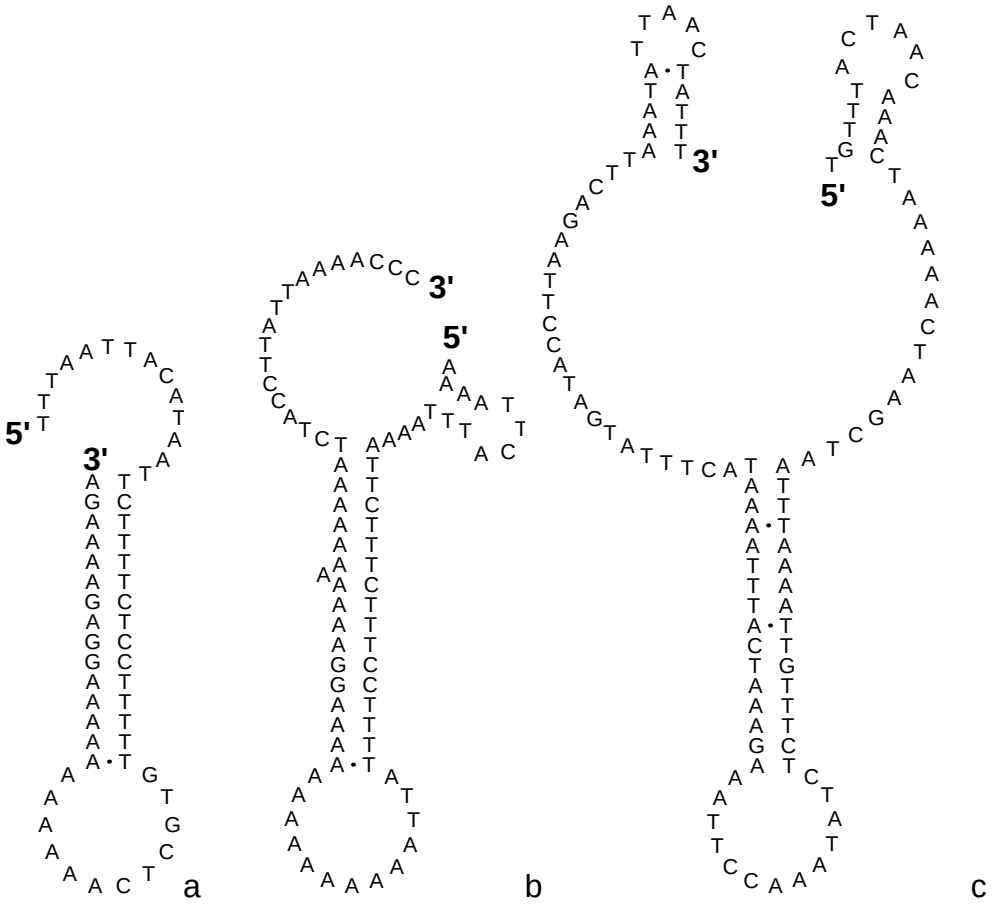
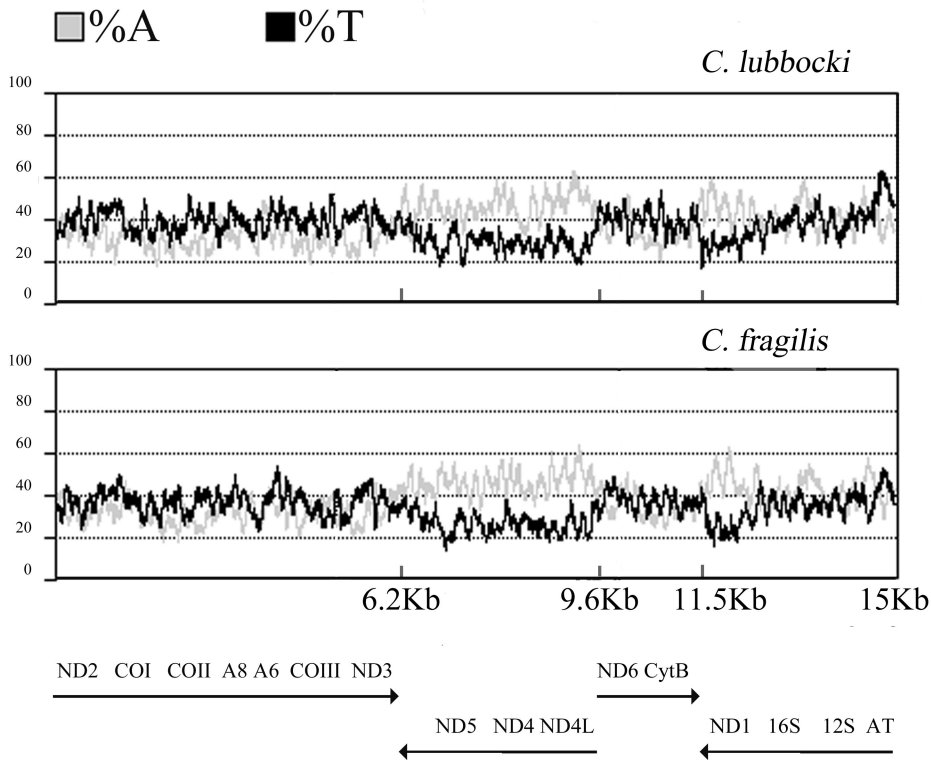
+5
-7
+80
-2

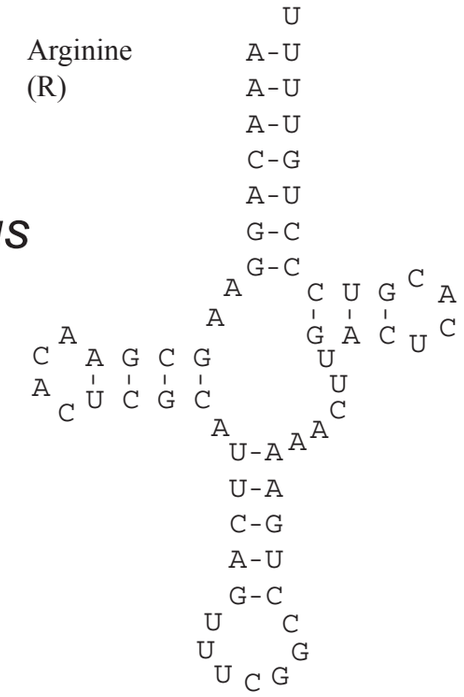

Campodea fragilis
I Q M
W C Y
L1
K
D
G
A
R
N
S1
E
F
H
P
T
L2
S2
V
nd2
cox1
cox2
atp8
atp6
cox3
nd3
nd5
nd4
nd4L
nd6
cob
nd1
lrRNA
srRNA
-3
+2
+111
+2
-1
-8
-2
-7
+4
-3
-2
-3
-2
-1
-2
-15
-1
-1
+2
+8
-7
+56
-2


5'
3'
a
3'
5'
b
3'
5'
c

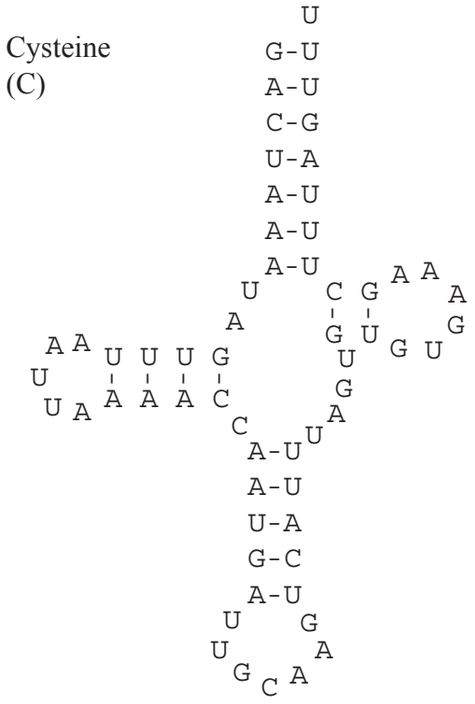

%A
%T
C. lubbocki
100
80
60
40
20
0
C. fragilis
100
80
60
40
20
0
6.2Kb
9.6Kb
11.5Kb
15Kb
ND2 COI COII A8 A6 COIII ND3
ND6 CytB
ND5 ND4 ND4L
ND1 16S 12S AT

Japyx solifugus

Arginine (R)



Cysteine (C)



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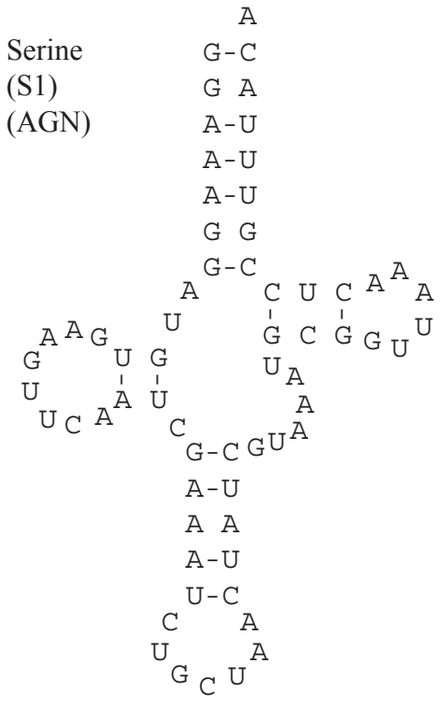

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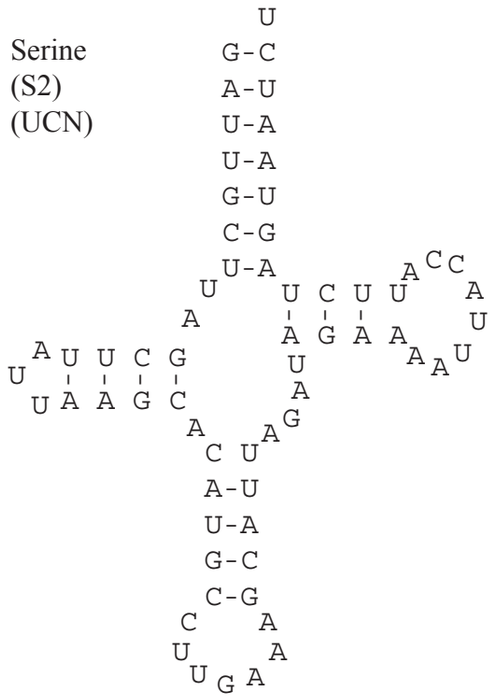
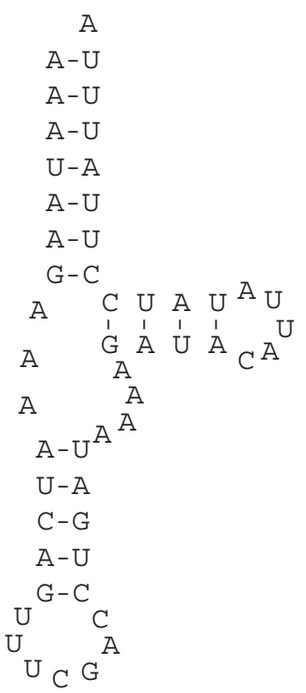


C. fragilis

Arginine (R)



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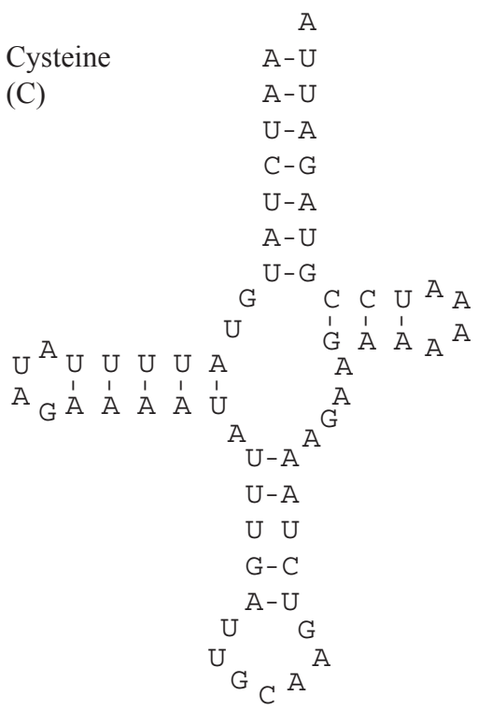

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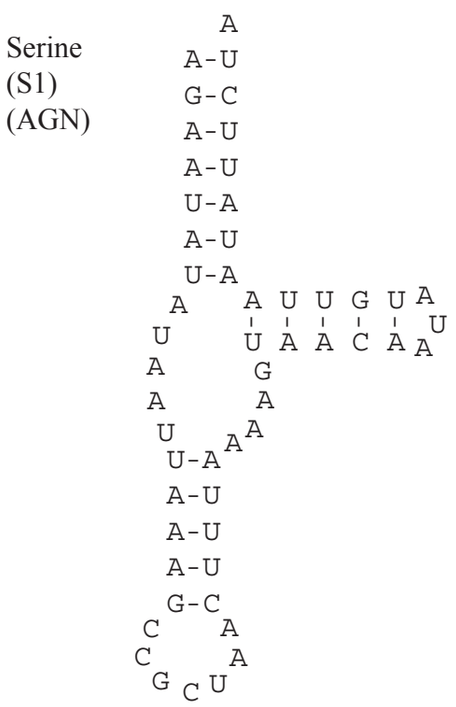

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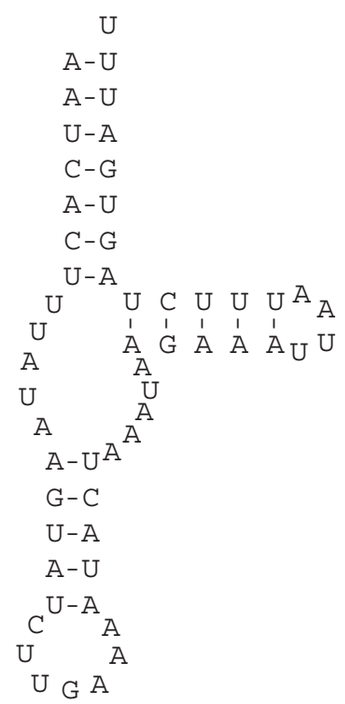
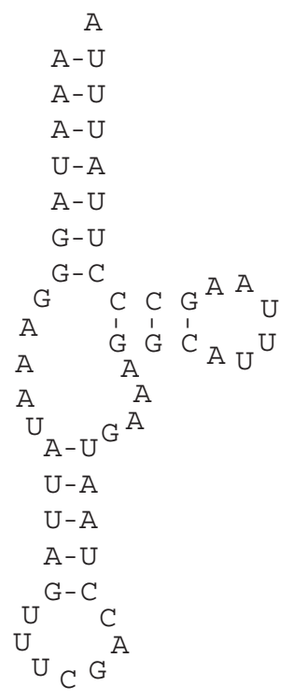


C. lubbocki

Arginine (R)



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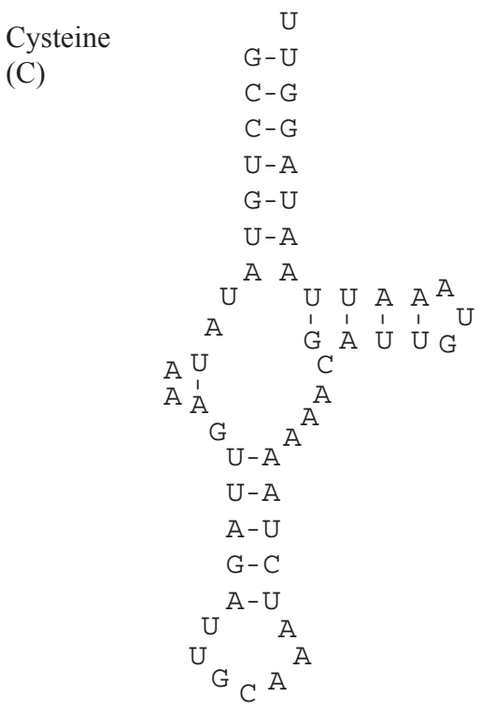

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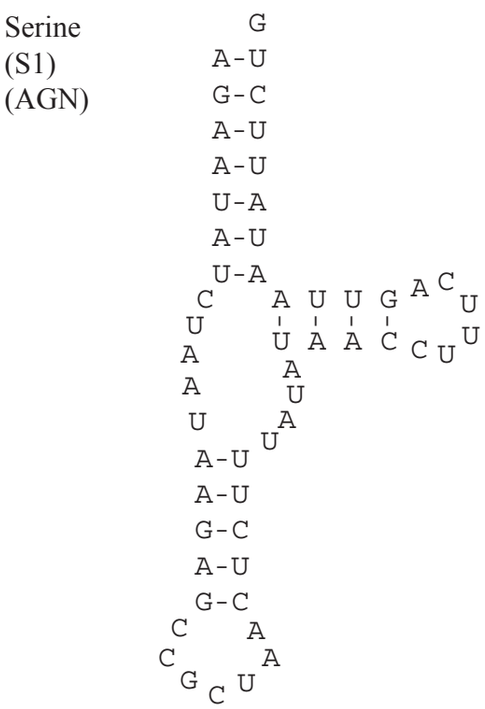

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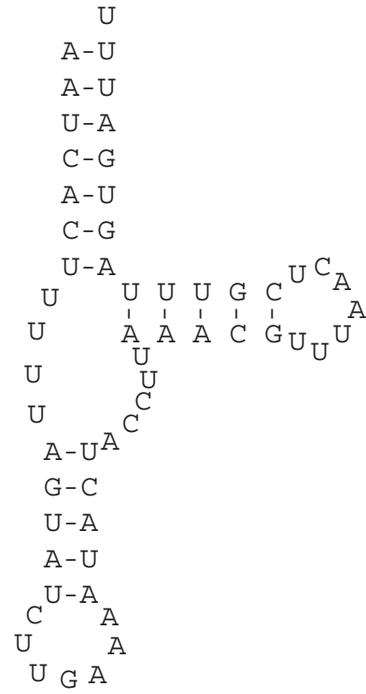
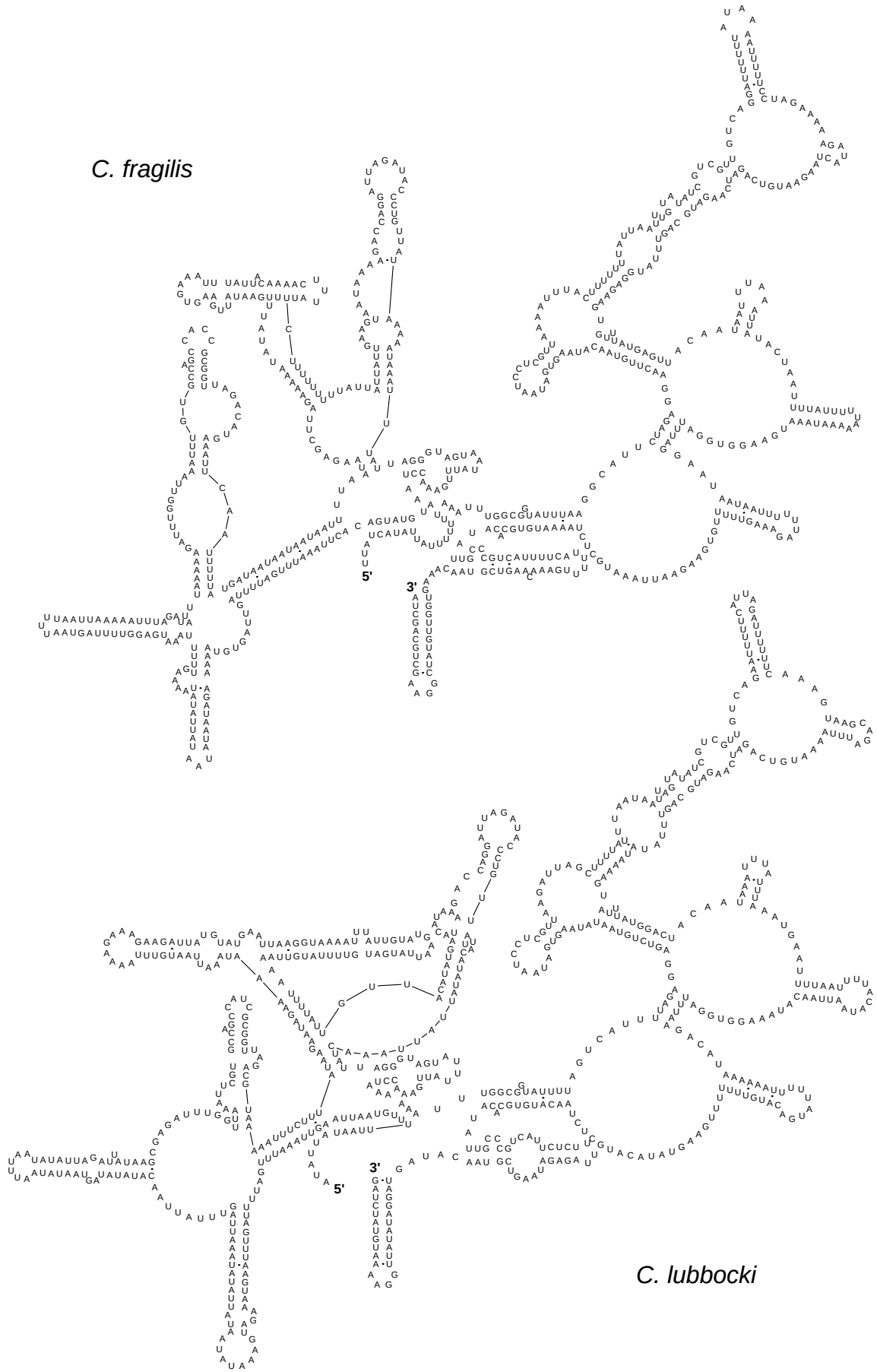
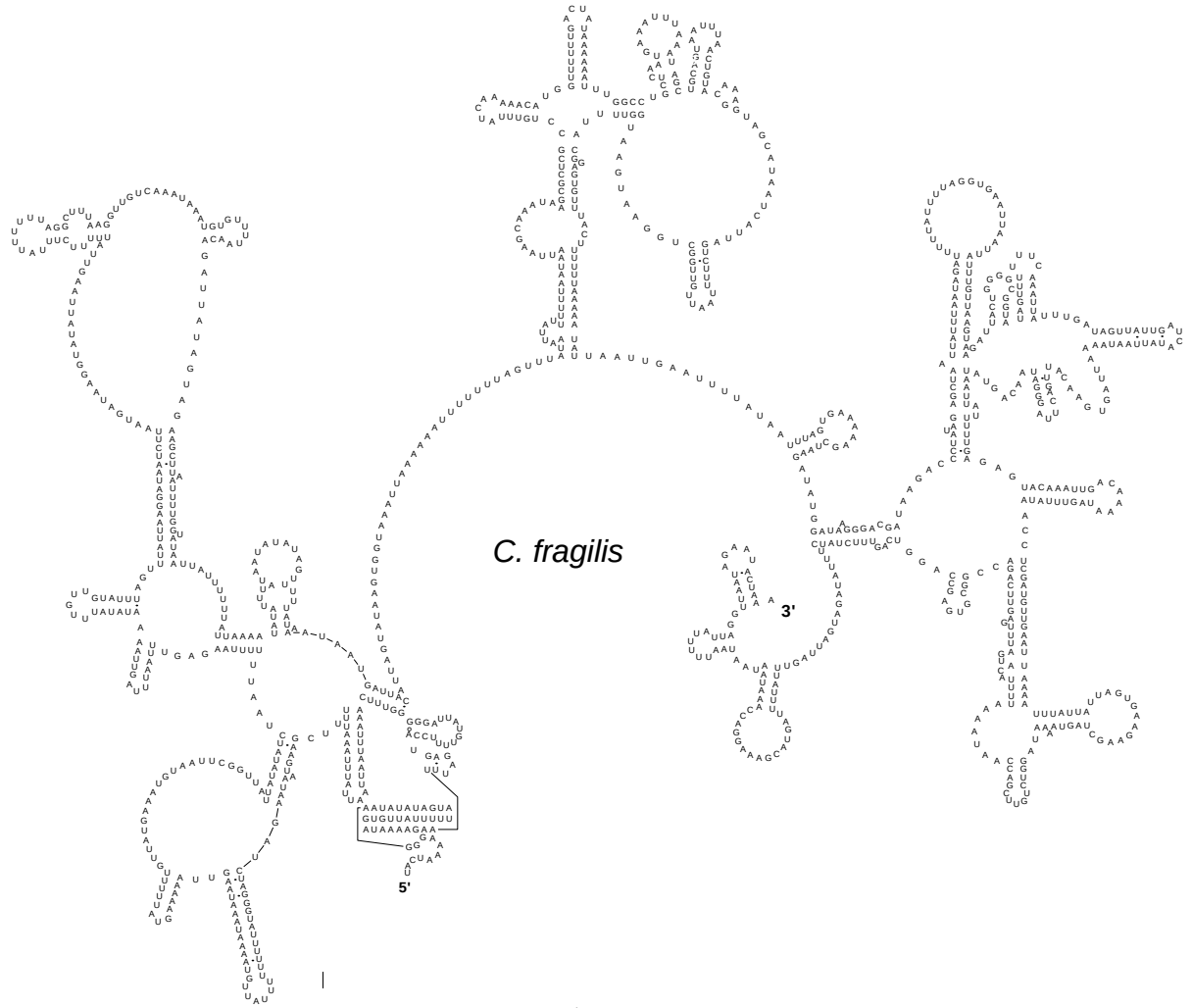
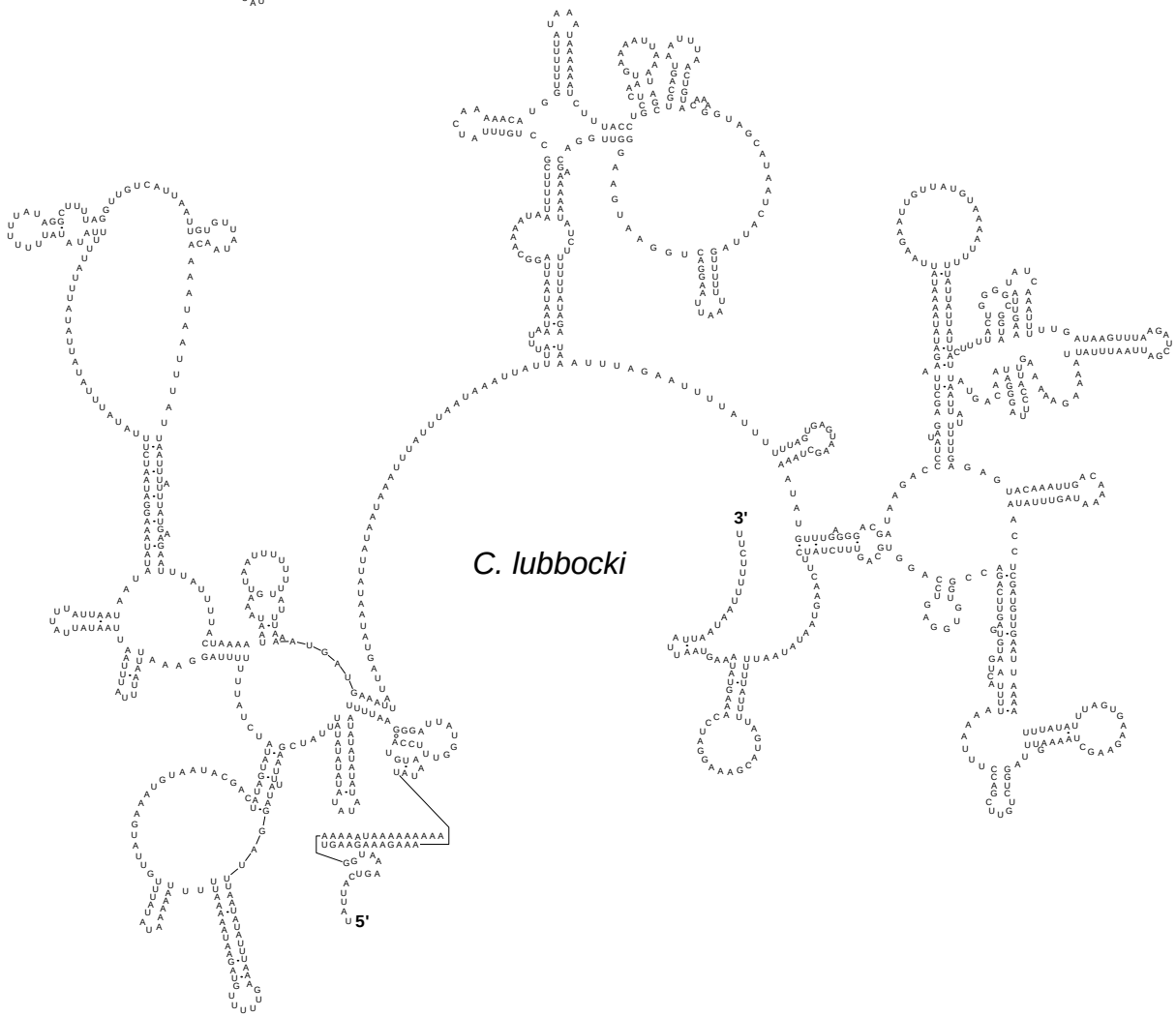



C. fragilis
5'
3'
3'
5'
C. lubbocki


C. fragilis
3'
5'


C. lubbocki
3'
5'

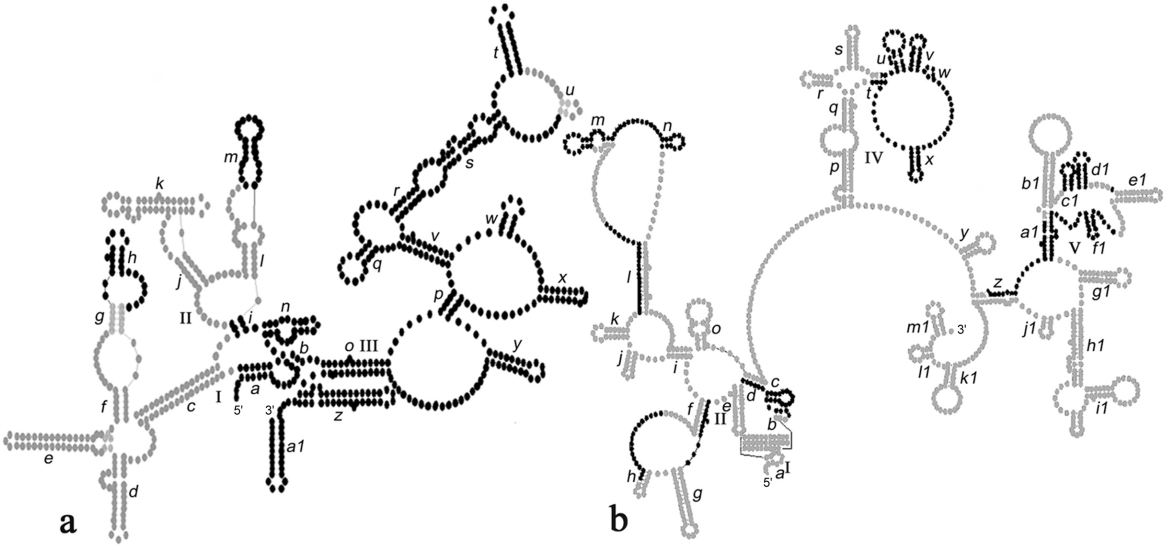

a
b
I
II
III
IV
V
5'
3'

Table 1

Higher ranking taxon	Species	Accession number	PCG	1st codon Position	2nd codon position	3rd codon position	rRNAs	tRNAs	total
Diplura	*Campodea fragilis*	XX	70.6	67.0	65.8	79.0	76.5	76.4	72.6
“	*Campodea lubbocki*	XX	73.0	68.3	66.9	84.2	80.3	76.1	74.9*
“	*Japyx solifugus*	AY771989	64.1	63.5	60.4	68.5	65.0	64.9	64.8
Collembola	*Gomphiocephalus hodgsoni*	NC005438	72.4	66.6	65.5	86.2	77.0	76.0	74.1
“	*Tetrodontophora bielanensis*	NC002735	71.3	66.4	66.2	81.4	77.0	75.5	72.7
“	*Podura aquatica*	NC006075	64.7	60.0	64.1	70.0	-	70.5	-
“	*Onychiurus orientalis*	NC006074	68.5	71.5	65.8	66.2	-	73.2	-
Archaeognatha	*Petrobius brevistylis*	AY956355	66.2	61.1	63.2	74.2	71.7	70.6	67.3
“	*Nesomachilis australica*	NC006895	67.2	60.8	64.4	76.8	72.6	70.3	68.8
Zygentoma	*Tricholepidion gertschi*	NC005437	67.7	63.0	63.0	77.1	71.2	69.0	68.6
“	*Thermobia domestica*	NC006080	66.3	59.4	62.9	76.6	67.9	67.2	67.0
Pterygota	*Locusta migratoria*	NC001712	74.1	68.9	66.1	87.3	77.7	74.0	75.3
“	*Drosophila yakuba*	X03240	76.7	69.8	66.4	93.7	81.9	76.6	78.6
“	*Apis mellifera*	L06178	83.3	79.3	75.4	95.2	83.4	87.1	84.9

* incomplete genome data; missing sequence of control region or control region and rRNAs respectively

Table 2

Gene (Strand)	identity (%)	*C. fragilis*						*C. lubbocki*					
		length	start/stop	%A	%C	%G	%T	length	start/stop	%A	%C	%G	%T
atp6 (+)	73.3	675	ATG/TAA	31.3	20.6	10.7	37.5	675	ATG/TAA	30.7	18.4	9.6	41.3
atp8 (+)	57.0	156	ATT/TAA	38.5	14.7	4.5	42.3	156	ATC/TAA	35.3	18.0	5.8	41.0
cox1 (+)	77.4	1540	ATT/T - -	29.0	19.7	15.7	35.7	1542	ATA/TAA	29.0	18.6	14.6	37.8
cox2 (+)	77.9	679	ATA/T - -	33.1	19.3	11.6	35.9	684	ATA/TAA	33.2	17.0	10.5	39.3
cox3 (+)	76.6	787	ATG/T - -	30.9	21.1	14.1	33.9	787	ATG/T - -	28.7	18.7	13.1	39.5
cob (+)	76.5	1143	ATG/TAA	32.8	19.5	11.2	36.5	1140	ATG/TAA	32.5	17.5	10.8	39.2
nad1 (-)	70.7	924	ATT/TAA	26.4	10.5	16.3	46.8	921	ATA/TAA	29.0	8.3	17.3	45.5
nad2 (+)	61.3	1005	ATA/TAA	32.1	20.2	9.2	38.5	1005	ATT/TAA	32.4	16.9	8.0	42.7
nad3 (+)	62.9	357	ATA/TAA	35.0	18.2	7.8	38.9	347	ATC/TA -	34.9	16.7	7.8	40.6
nad4 (-)	68.7	1338	ATG/TAA	25.7	8.2	17.6	48.5	1326	ATG/T - -	27.9	6.9	17.2	48.0
nad4l (-)	74.0	285	ATG/TAA	28.4	4.9	20.4	46.3	285	ATG/TAG	27.4	1.8	21.4	49.5
nad5 (-)	73.7	1707	ATA/TAA	26.5	8.0	19.5	46.0	1710	ATC/TAG	30.0	7.3	17.5	45.2
nad6 (+)	49.5	510	ATT/TAA	38.4	17.1	4.7	39.8	524	ATT/TA -	37.6	16.8	4.8	40.8

Table 3

		C.l.	*C.f.*	*J.s.*	*G.h.*	*T.b.*	*P.a.*	*O.o.*	*P.b.*	*N.a.*	*T.g.*	*T.d.*	*L.m.*
GCA	A	1.15	1.03	**1.68**	1.22	1.13	0.82	0.75	1.29	**1.66**	**1.70**	1.33	**2.11**
GCU	A	**2.18**	**1.87**	1.39	**2.17**	**2.00**	**1.83**	**2.55**	**1.42**	1.52	1.28	**1.46**	1.68
GCG	A	0.06	0.14	0.31	0.12	0.09	0.44	0.15	0.33	0.16	0.20	0.23	0.05
GCC	A	0.62	0.96	0.62	0.49	0.78	0.91	0.55	0.96	0.67	0.83	0.99	0.16
UGC	C	0.33	0.43	0.28	0.41	0.31	0.82	0.58	0.63	0.35	0.50	0.49	0.36
UGU	C	**1.67**	**1.57**	**1.72**	**1.59**	**1.69**	**1.18**	**1.42**	**1.38**	**1.65**	**1.50**	**1.51**	**1.64**
GAU	D	**1.46**	**1.40**	**1.09**	**1.53**	**1.44**	**1.22**	**1.20**	**1.44**	**1.18**	**1.32**	**1.49**	**1.68**
GAC	D	0.54	0.60	0.91	0.47	0.56	0.78	0.80	0.56	0.82	0.69	0.51	0.33
GAG	E	0.51	0.58	0.63	0.41	0.49	0.55	0.56	0.71	0.49	0.47	0.54	0.29
GAA	E	**1.49**	**1.42**	**1.37**	**1.59**	**1.51**	**1.45**	**1.44**	**1.29**	**1.51**	**1.53**	**1.46**	**1.71**
UUU	F	**1.75**	**1.66**	**1.30**	**1.80**	**1.65**	**1.50**	**1.66**	**1.59**	**1.67**	**1.49**	**1.32**	**1.49**
UUC	F	0.25	0.34	0.70	0.20	0.35	0.50	0.34	0.41	0.33	0.51	0.68	0.52
GGU	G	1.21	**1.48**	1.39	0.87	1.02	0.71	0.96	1.27	1.23	1.22	1.24	1.77
GGG	G	1.00	0.66	0.71	1.01	0.90	**1.87**	1.27	0.82	0.80	0.98	0.51	0.19
GGC	G	0.11	0.41	0.42	0.21	0.17	0.38	0.34	0.45	0.55	0.20	0.83	0.02
GGA	G	**1.68**	1.45	**1.48**	**1.92**	**1.91**	1.03	**1.43**	**1.46**	**1.43**	**1.60**	**1.42**	**2.02**
CAC	H	0.35	0.84	**1.19**	0.32	0.73	**1.11**	0.49	0.86	0.87	0.78	0.80	0.70
CAU	H	**1.65**	**1.17**	0.81	**1.68**	**1.27**	0.89	**1.51**	**1.14**	**1.13**	**1.23**	**1.21**	**1.30**
AUU	I	**1.75**	**1.61**	**1.46**	**1.77**	**1.65**	**1.49**	**1.48**	**1.49**	**1.63**	**1.59**	**1.63**	**1.78**
AUC	I	0.25	0.39	0.54	0.23	0.35	0.52	0.52	0.51	0.37	0.41	0.37	0.23
AAA	K	**1.64**	**1.60**	**1.22**	**1.64**	**1.44**	**1.44**	**1.58**	**1.41**	**1.45**	**1.51**	**1.21**	**1.41**
AAG	K	0.36	0.40	0.78	0.36	0.56	0.56	0.42	0.59	0.55	0.49	0.80	0.59
UUG	L	0.53	0.70	1.67	0.31	0.64	0.53	0.81	0.86	1.07	1.00	0.56	0.54
UUA	L	**3.77**	**3.50**	**1.93**	**4.04**	**3.39**	**2.54**	**2.85**	**2.62**	**2.46**	**2.65**	**2.61**	**3.91**
CUA	L	0.56	0.66	1.49	0.64	1.07	1.23	0.81	1.17	1.08	1.28	1.18	0.83
CUC	L	0.24	0.28	0.11	0.08	0.12	0.38	0.25	0.18	0.33	0.16	0.44	0.06
CUG	L	0.08	0.07	0.25	0.04	0.07	0.31	0.21	0.26	0.13	0.23	0.13	0.05
CUU	L	0.81	0.79	0.55	0.89	0.71	1.02	1.08	0.90	0.93	0.69	1.09	0.61
AUG	M	0.27	0.51	0.46	0.23	0.23	0.51	0.38	0.44	0.32	0.45	0.43	0.31
AUA	M	**1.73**	**1.49**	**1.54**	**1.78**	**1.77**	**1.49**	**1.62**	**1.56**	**1.68**	**1.55**	**1.57**	**1.69**
AAC	N	0.31	0.56	1.00	0.41	0.51	0.80	0.66	0.66	0.64	0.46	0.59	0.48
AAU	N	**1.69**	**1.44**	**1.00**	**1.59**	**1.49**	**1.20**	**1.34**	**1.34**	**1.36**	**1.54**	**1.41**	**1.52**
CCU	P	**2.06**	1.38	1.58	**2.05**	**1.80**	**1.70**	**2.05**	**1.67**	**1.70**	0.94	**1.83**	1.50
CCG	P	0.17	0.32	0.15	0.09	0.15	0.19	0.18	0.36	0.19	0.14	0.18	0.09
CCC	P	0.78	0.43	0.59	0.39	0.46	0.99	0.43	0.93	0.69	0.61	0.85	0.12
CCA	P	0.99	**1.87**	**1.68**	1.47	1.59	1.12	1.34	1.04	1.43	**2.31**	1.14	**2.29**
CAG	Q	0.20	0.30	0.49	0.18	0.14	0.40	0.26	0.37	0.54	0.27	0.23	0.10
CAA	Q	**1.80**	**1.71**	**1.51**	**1.82**	**1.86**	**1.60**	**1.74**	**1.63**	**1.46**	**1.73**	**1.77**	**1.91**
CGA	R	**2.25**	**2.59**	**1.31**	**1.78**	**1.71**	**1.39**	1.47	**2.26**	1.42	**1.93**	**2.03**	**2.47**
CGC	R	0.17	0.31	0.30	0.44	0.33	0.77	0.33	0.26	0.14	0.21	0.49	0.07
CGG	R	0.00	0.31	0.48	0.07	0.65	0.77	0.41	0.45	0.68	0.83	0.62	0.15
CGU	R	1.58	0.78	1.91	1.70	1.31	1.08	**1.80**	1.03	**1.76**	1.03	0.86	1.31
AGC	S	0.13	0.40	0.33	0.30	0.16	0.47	0.29	0.40	0.43	0.17	0.30	0.07
AGU	S	0.61	0.87	1.18	0.91	1.03	0.99	0.73	1.10	0.86	0.95	1.28	0.53
AGG	S	0.23	0.23	0.18	0.04	0.16	0.09	0.00	0.07	0.02	0.30	0.03	0.04
UCU	S	2.09	2.30	1.72	**2.82**	**2.43**	**2.66**	**2.67**	**1.93**	**2.56**	1.86	**2.40**	**2.69**
UCG	S	0.13	0.11	0.23	0.11	0.16	0.18	0.31	0.47	0.26	0.11	0.20	0.09
AGA	S	1.73	1.23	0.90	1.46	1.10	1.42	1.40	1.87	1.90	1.46	1.38	1.79
UCC	S	0.71	0.40	0.72	0.47	1.06	0.90	0.94	0.81	0.64	0.68	0.69	0.15
UCA	S	**2.38**	**2.45**	**2.74**	1.89	1.91	1.29	1.65	1.35	1.33	**2.48**	1.73	2.65
ACA	T	1.63	**1.64**	**1.92**	1.40	1.27	1.21	1.07	**1.52**	**1.69**	**1.93**	**1.68**	**2.56**
ACU	T	**1.65**	1.61	1.13	**2.28**	**1.93**	**1.79**	**2.01**	**1.52**	1.43	1.18	1.54	1.15
ACC	T	0.67	0.73	0.74	0.30	0.73	0.74	0.78	0.75	0.76	0.80	0.68	0.23
ACG	T	0.05	0.02	0.22	0.02	0.07	0.27	0.15	0.21	0.12	0.09	0.09	0.06
GUU	V	**1.76**	**1.51**	1.30	**1.83**	1.54	**1.50**	**1.44**	1.45	**1.69**	**1.56**	1.46	**2.08**
GUG	V	0.29	0.48	0.82	0.42	0.28	0.92	0.74	0.57	0.47	0.48	0.35	0.07
GUA	V	1.70	1.49	**1.78**	1.60	**1.96**	1.16	1.33	**1.55**	1.54	**1.56**	**1.91**	1.75
GUC	V	0.25	0.53	0.11	0.15	0.22	0.43	0.49	0.43	0.30	0.41	0.28	0.11
UGA	W	**1.55**	**1.77**	**1.39**	**1.62**	**1.72**	**1.65**	**1.30**	**1.65**	**1.54**	**1.71**	**1.70**	**1.92**
UGG	W	0.45	0.23	0.61	0.38	0.28	0.35	0.70	0.36	0.46	0.29	0.31	0.08
UAU	Y	**1.67**	**1.54**	**1.30**	**1.54**	**1.47**	**1.12**	**1.44**	**1.35**	**1.40**	**1.48**	**1.46**	**1.62**
UAC	Y	0.33	0.46	0.70	0.46	0.53	0.88	0.56	0.65	0.60	0.52	0.54	0.38

Bolded numbers represent the codon most commonly used to code the amino acid. *C.f.= Campodea fragilis, C.l.= C. lubbocki, J.s.= Japyx solifugus, G.h.=Gomphiocephalus hodgsoni, T.b.= Tetrodontophora bielanensis, P.a.= Podura aquatica, O.o.= Onychurus orientalis, P.b.= Petrobius brevistylis, N.a.= Nesomachilis australica, T.g.= Tricholepidion gertschi, T.d.= Thermobia domestica, L.m.= Locusta migratoria*